REMARKS

Status of the Claims

Claims 20, 21, 27, 28, 30, 44, 46, 50 and 51 are currently pending in the application. Claims 20, 21, 23, 24, 26-28, 30 and 44-46 stand rejected. Claims 20 and 27 have been amended as set forth herein. Claims 23, 24, 26 and 45 have been cancelled herein. All cancellations and amendments are made without prejudice or disclaimer. New claims 50 and 51 have been added herein. No new matter has been added by way of the present amendments. Specifically, the subject matter of new claims 50 and 51 are supported by the specification at page 17, lines 21-25. Changes to claims 20 and 27 are supported by the as-filed claims. Reconsideration is respectfully requested.

Rejections Under 35 U.S.C. § 103(a)

Gelfand et al. (U.S. Patent No. 5,693,517) and Kaiser et al. (U.S. Patent No. 5,843,669)

Claims 20, 21 and 44 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Gelfand et al., U.S. Patent No. 5,693,517 (hereinafter, "Gelfand et al.") in view of Kaiser et al., U.S. Patent No. 5,843,669 (hereinafter, "Kaiser et al."). (*See*, Office Action of February 28, 2006, at page 2, hereinafter "Office Action"). Applicants traverse the rejection as hereinafter set forth.

M.P.E.P. § 706.02(j) sets forth the standard for establishing a *prima facie* case of obviousness as follows:

> To establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or combine reference teachings. Second, there must

> be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on applicant's disclosure. *In re Vaeck*, 947 F.2d 488, 20 U.S.P.Q.2d 1438 (Fed. Cir. 1991).

The Examiner states that Gelfand et al. disclose using hydroxymethyl dUTP or 7-deaza-dGTP in generating a first strand cDNA in an RT-PCR reaction. (*Id.* at page 2). The Examiner further states that Kaiser et al. disclose a PCR reaction wherein 7-deaza-dATP and 7-deaza-dGTP are used. (*Id.*). As to the disclosure of Gelfand et al., the Examiner points out additionally that several embodiments are disclosed directed at suppressing the cross-contamination from positive control templates or from previous amplification reactions wherein the embodiments may use other modified nucleoside triphosphates capable of effecting the T_m of DNA product, such as c7dGTP, 7-deaza-dGTP or α-phosphorothiolate dNTPs. (*Id.* at pages 3-4). The Examiner also finds motivation to combine the references and so modify them to arrive at the previously claimed invention in Kaiser et al. wherein the reasoning behind use of the nucleoside analogs is described at column 183, lines 1-8.

However, claim 20 recites, "A method for amplifying a DNA, comprising the steps of (a) preparing a cDNA comprising <u>at least two kinds of nucleotide analogs</u> by a reverse transcription reaction using an RNA as a template <u>in the presence of 7-Deaza-dATP and at least one nucleotide analog selected from the group consisting of 7-Deaza-dGTP and dITP</u>; and (b) amplifying a desired DNA from the cDNA obtained in the above step (a), in the presence of two or more kinds of nucleotide analogs, wherein said nucleotide analogs are 7-Deaza-dATP and at least one nucleotide analog is selected from the group consisting of 7-Deaza-dGTP and dITP,

wherein the nucleotide analogs are uniformly incorporated into the resulting DNA and do not cause termination of the amplification, thereby selectively amplifying DNA of a target sequence derived from RNA."

To support a *prima facie* case of obviousness, the references must disclose or suggest each and every element of the presently claimed invention. (*See*, *In re Vaeck*). Gelfand et al. and Kaiser et al., either alone or in combination, do not disclose or suggest each and every limitation of the presently claimed invention, especially they do not disclose or suggest the use of two kinds or more of nucleotide analogs simultaneously either in an RT-PCR reaction, or PCR reaction, as recited in dependent claim 21. The presently claimed invention has been clarified to emphasize this limitation, as recited in claims 20 and 27. The present inventors have fortuitously found that by using two or more kinds of analogs simultaneously, the present invention achieves higher amplification efficiency as compared to reactions using only one single nucleotide analog. (*See*, for example, as-filed specification, at Example 3, pages 27-29).

Neither do the references, individually, or in combination, disclose or suggest the use of the two specific nucleotide analogs, 7-Deaza-dGTP and 7-Deaza-dATP, as recited in claim 44, simultaneously in one reaction, either PCR or RT-PCR.

Furthermore, Gelfand et al. do not disclose or suggest incorporating 7-deaza-dATP in reverse transcription reactions, as required in the presently claimed invention. A person of ordinary skill in the art would not have had a reasonable expectation of success in using this particular analog in such a reaction since although it is known 7-deaza-dATP may be a substrate for DNA polymerase, it was not known as of the filing date of the present application whether this analog could also be used as a substrate for reverse transcriptase in a DNA synthesis

reaction. (*See*, for example, Ide et al., *Nucleic Acids Research*, 16(23):11339-11354, 1988, wherein it is disclosed that although the nucleotide analog dihydrothymidine-dTTP was capable of acting as a substrate for *E. coli* DNA pol I, reverse transcriptase did not use it as a substrate, complementary copy of reference attached hereto as Exhibit A).

Thus, a *prima facie* case of obviousness has not been established since the two references, either individually or in combination, do not disclose or suggest each and every element of the presently claimed invention. Furthermore, there is no motivation found within the references or within the knowledge of one of ordinary skill in the art to suggest modification of the cited references to arrive at the presently claimed invention comprising using two or more kinds of nucleotide analogs in either RT-PCR or PCR. (*See*, *In re Vaeck*).

Reconsideration and withdrawal of the obviousness rejection of claims 20, 21 and 44 are respectfully requested.

<u>Gelfand et al., Kaiser et al. & Pergolizzi et al. (U.S. Patent No. 5,658,764)</u>

Claims 23, 24, 26-28, 30, 45 and 46 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over Gelfand et al. in view of Kaiser et al. and Pergolizzi et al., U.S. Patent No. 5,658,764 (hereinafter "Pergolizzi et al."). (*See*, Office Action, at page 6). Claims 23, 24, 26 and 45 have been cancelled herein without prejudice or disclaimer, thus obviating the rejection as to these claims. Applicants traverse the rejection as to claims 27, 28, 30 and 46 as hereinafter set forth.

In addition to that already discussed, above, with respect to the disclosures of Gelfand et al. and Kaiser et al., the Examiner states that Pergolizzi et al. disclose using 7-deaza-dGTP and DMSO

in their PCR experiments. (*Id.*). Claim 27 recites, "A method for amplifying a DNA comprising the steps of: (a) preparing a cDNA by a reverse transcription reaction using RNA as a template in the presence of 7-Deaza-dATP and at least one nucleotide analog selected from the group consisting of 7-Deaza-dGTP and dITP; and (b) amplifying a desired DNA from the cDNA of the above step (a) in the presence of the following substances (i) to (iii): (i) at least one nucleotide analog selected from the group consisting of 7-Deaza-dGTP and dITP, (ii) 7-Deaza-dATP, and (iii) a compound for lowering the Tm value of a double-stranded nucleic acid, wherein the nucleotide analogs (i) and (ii) are uniformly incorporated into the resulting DNA, thereby selectively amplifying DNA of a target sequence derived from RNA."

As commented upon, above, the references, either individually or in combination, do not disclose or suggest the use of the two specific nucleotide analogs, 7-Deaza-dATP and 7-Deaza-dGTP, as recited in claim 27. Claim 28 depends from claim 27 and further limits claim 27 to the use of the method in PCR experiments. Claim 30 further narrows the compound for lowering the Tm value to a compound selected from the group consisting of: formamide, dimethyl sulfoxide and trimethyl glycine. Claim 46 further recites, in part, "wherein both of 7-Deaza-dGTP and 7-Deaza-dATP are used in step (a) and (b) as the nucleotide analogs." The arguments presented above, with respect to the inadequacies of the disclosures of Gelfand et al. and Kaiser et al. are incorporated herein by reference and are relied upon as above to rebut the present rejection of claims 27, 28, 30 and 46.

Thus, a *prima facie* case of obviousness has not been established since the two references, either individually or in combination, do not disclose or suggest each and every element of the presently claimed invention. Furthermore, there is no motivation found within the references or

within the knowledge of one of ordinary skill in the art to suggest modification of the cited references to arrive at the presently claimed invention comprising using two or more kinds of nucleotide analogs in either RT-PCR or PCR. (*See*, *In re Vaeck*).

Reconsideration and withdrawal of the obviousness rejection of claims 27, 28, 30 and 46 are respectfully requested.

CONCLUSION

If the Examiner has any questions or comments, please contact Thomas J. Siepmann, Registration No 57,374 at the offices of Birch, Stewart, Kolasch & Birch, LLP.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to our Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. § 1.16 or under § 1.17; particularly, extension of time fees.

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Respectfully submitted,

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Dihydrothymidine and thymidine glycol triphosphates as substrates for DNA polymerases: differential recognition of thymine C5-C6 bond saturation and sequence specificity of incorporation

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ABSTRACT

The ability of dihydrothymidine (DHdTTP) and thymidine glycol (dTTP-GLY) 5'-triphosphates to serve as substrates for different DNA polymerases was investigated. DHdTTP but not dTTP-GLY was used as a substrate by *E. coli* DNA polymerase I (Pol I). Within the detection limit of the assay used, neither T4 DNA polymerase nor avian myeloblastosis virus (AMV) reverse transcriptase used DHdTTP or dTTP-GLY as substrates. The ability of DHdTTP and dTTP-GLY to undergo enzyme-catalyzed turnover to the monophosphate paralleled their ability to serve as substrates for polymerization. These results, along with kinetic parameters for the incorporation of DHdTTP with Pol I, strongly suggest that the saturation of thymine C5-C6 bond and the substituent groups at C5 and C6 differentially exert effects on binding to DNA polymerases. DNA sequencing gel analysis of the polymerization products revealed that most single adenine sites were capable of templating DHdTTP, however, DNA synthesis was partially arrested at multiple adenine sites, suggesting that sequential incorporation of DHdTTP produced significant disorder in the primer terminus.

INTRODUCTION

Genotoxic agents that produce free radicals generate a spectrum of DNA damages. These agents include ionizing radiation (1,2), 1 MHz ultrasound used in medical diagnostics (3), hydroperoxides (4,5), and certain antibiotics (6,7). One of the difficulties in assessing the biological consequences of a particular damage produced by free radicals is that very few methods are available to introduce a unique damage into DNA. In a previous paper we described an attempt to engineer stable altered DNA bases such as 5,6-dihydrothymine and thymine glycol (5,6-dihydroxy-5,6-dihydrothymine) into DNA (8). Our approach was to chemically synthesize the nucleoside triphosphates and to use them as substrates for *Escherichia coli* DNA polymerase I Klenow fragment. A similar approach has been successfully applied by Singer *et al.* (9,10) and Preston *et al.* (11) for the preparation of DNA and polynucleotides containing O_4-alkylated thymines. We chose dihydrothymidine (DHdTTP) and thymidine glycol (dTTP-GLY) 5'-triphosphates (Fig. 1) as substrates because saturation of C5-C6 bond and concomitant loss of planarity of the pyrimidine

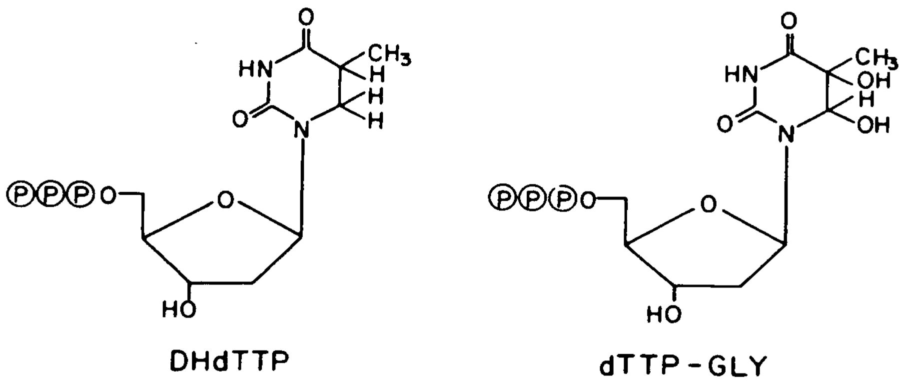

Figure 1. Structures of DHdTTP and dTTP-GLY.

ring are considered to be notable characteristics of the lesions induced by free radicals such as ·OH and H· (1,12,13). Further, it has been found that thymine glycol in the DNA template retains coding ability (14-17). Osmium tetroxide and potassium permanganate have been widely used to produce thymine glycol in DNA, however, these reagents also produce additional minor base damages (18,19) as well as strand breaks (Ide *et al.* unpublished results). In a previous study we found that, although the loss of planarity in the thymine ring is a common feature for both DHdTTP and dTTP-GLY, surprisingly, only DHdTTP was incorporated into DNA by Pol I Klenow fragment.

We report here evidence which strongly suggests that DHdTTP and dTTP-GLY are differentially recognized by DNA polymerases from different origins, and that although dihydrothymine has a nonplanar ring structure, it does not produce significant disorder in the newly synthesized strand unless it is multiply incorporated. These data are interesting not only from the viewpoint of studying the biological consequences of altered DNA bases but also for elucidating the mechanism of fidelity of DNA polymerases as it relates to their initial interaction with nucleoside triphosphates.

MATERIALS AND METHODS

Chemicals

HPLC-purified deoxyribonucleoside triphosphates (dNTP) were obtained from P-L Biochemicals. dTMP and acid molybdate spray reagent were from Sigma. Nucleoside 5′-monophosphates of dihydrothymidine (DHdTMP) and thymidine glycol

(dTMP-GLY) were synthesized following the methods of Cohn and Doherty (20) and Rajagopalan *et al.* (21), respectively. PEI-cellulose TLC plates were from Macherey-Nagel. [^{3}H-methyl]dTTP (70 Ci/mmol), [5-^{3}H]dCTP (28 Ci/mmol), [8-^{3}H]dATP (22 Ci/mmol), and [γ-^{32}P]ATP (~3000 Ci/mmol) were from ICN. [^{3}H]dTTP-GLY was prepared following the method of Cadet and Teoule (22). Briefly, an equivolume of mixture of water and pyridine (total 40 ul) containing 200 uCi of [^{3}H]dTTP and 40 mM of bromine was incubated at 50°C for 1 hour and the reaction solution evaporated. Evaporation was repeated after adding an aliquot of water, then the crude [^{3}H]dTTP-GLY was subjected to HPLC purification. [^{3}H]DHdTTP was prepared as previously described (8). The HPLC purification of [^{3}H]DHdTTP and [^{3}H]dTTP-GLY, and their conversion to triethylammonium salts, were essentially the same as described before (8) except that an analytical HPLC column SOTA AX 100 (4 x 250 mm) was used instead of a preparative one. Final yields of ^{3}H-labeled triphosphates were about 10%. The structures of [^{3}H]DHdTTP and [^{3}H]dTTP-GLY were confirmed by alkaline phosphatase digestion (8). The maximum contamination of dTTP in the final preparations of unlabeled and ^{3}H-labeled DHdTTP and dTTP-GLY after HPLC purification was estimated as 1 part per 10^4-10^5. Thus we could eliminate possible artifacts that might be due to the contamination of the modified dTTP preparations by dTTP.

Enzymes and DNA

Escherichia coli DNA polymerase I (Pol I) and its large fragment (Klenow), phage T4 DNA polymerase and avian myeloblastosis virus (AMV) reverse transcriptase were obtained from Pharmacia. Phage T4 polynucleotide kinase and restriction endonuclease *AvaII* were from New England BioLabs. Poly(dA-dT) [MW (1-5) x 10^5] was obtained from Sigma. M13mp11 DNA template primed with synthetic 17-mer [New England BioLabs, 5'-d(GTAAAACGACGGCCAGT)] (14) and PM2 DNA (23) were prepared as described. Restriction digestion by *AvaII* of PM2 DNA, having a unique recognition site (24), was carried out as recommended by the supplier. The linearized PM2 DNA was isolated by phenol extraction and ethanol precipitation.

Ability of modified nucleotides to replace dTTP

The primer annealed to M13mp11 template (0.77 ug) in 15 ul of polymerization buffer was elongated at 25°C by different DNA polymerases in the presence of 10 uM each of the three normal dNTP [A,G, and C (1.5 x 10^4 cpm/pmol)] and 10 uM dTTP or a modified nucleotide. Unless otherwise noted, the DNA polymerization buffer contained 50 mM Tris-HCl (pH 8.0), 5 mM dithiothreitol and 8 mM $MgCl_2$. For each reaction, 1.5 units of Pol I or

Klenow fragment, 3 units of T4 DNA polymerase, or 6.2 units of AMV reverse transcriptase were used. Samples (3 ul) of reaction mix were removed at given times (2,4,6 min) and were analyzed by precipitation with 5% TCA and filtration on Whatman GF/A filters. The extent of primer elongation as measured by [^{3}H]dCTP incorporation was corrected by subtraction of the background in a control polymerization with the three normal nucleotides (A,G,C) alone.

<u>Determination of kinetic parameters</u>

For the determination of kinetic parameters of DNA synthesis with dTTP or DHdTTP, poly(dA-dT) (10 ug) in 100 ul of polymerization buffer was replicated by Pol I Klenow fragment (2.5 units, 8220 units/mg) in the presence of dTTP or DHdTTP (0.5-10 uM) and [^{3}H]dATP (100 uM, 375 cpm/pmol) at 25°C. At appropriate incubation times (1,2,3 min), 30 ul of the reaction mix was removed and the radioactivity incorporated into poly(dA-dT) was assayed by precipitation with 5% TCA and filtration on Whatman GF/A filters. The data were corrected by subtraction of the background obtained in a control polymerization with dATP alone.

<u>Turnover of modified nucleotides</u>

DNA polymerase catalyzed turnover of DHdTTP and dTTP-GLY to monophosphates was assayed using *AvaII* digested PM2 DNA ends as templates (see also Fig. 3). Incubation was carried out at 25°C in 12 ul of polymerization buffer containing digested PM2 DNA (0.5 ug), 1.5 units of Pol I Klenow fragment or 5 units of T4 DNA polymerase, 1 uM each of dATP, dGTP and ^{3}H-labeled dTTP or a modified nucleotide. The specific activity was 4.4 x 10^4 cpm/pmol for dTTP and DHdTTP, and 1.3 x 10^4 cpm/pmol for dTTP-GLY. Samples (3 ul) were removed at given times (30 and 60 min) and spotted on PEI-cellulose TLC plates which had been prespotted with EDTA and dTMP as a marker. The plates were developed with 0.6 M LiCl. In a separate experiment using unlabeled mono and triphosphates, we found that the R_f-values of the monophosphates were close to each other [dTMP (0.52), DHdTMP (0.56), dTMP-GLY (0.60)]. Therefore we used only dTMP as a marker that could be conveniently located by UV absorption. The R_f-values of DHdTMP and dTMP-GLY were determined by acid molybdate reagent. The monophosphates were completely separated from the corresponding triphosphates which were retained close to the origin under these conditions. The developed plates were air dried and cut into small pieces and the radioactivity of each piece was determined.

<u>Product analysis with DNA sequencing gels</u>

The ability of modified nucleotides to replace either of the normal 4

dNTPs, and the sequence specificity of incorporation were assayed using the method developed by Revich *et al.* (25). 50 pmol of 17-mer was 5'-end labeled with [γ-^{32}P]ATP (50 pmol, 150 uCi) and 20 units of polynucleotide kinase. The reaction buffer (50 ul) consisted of 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine. After incubation for 30 min at 37°C, the reaction solution was subjected to mini C_{18} column (ca. 100 ul) and the column was washed with water (400 ul), followed by 25% acetonitrile (400 ul). The acetonitrile fractions containing the primer were evaporated to dryness. The aqueous solution containing M13mp11 template, 5'-end-labeled primer (5-fold molar excess of template), 400 mM NaCl, 50 mM Tris-HCl (pH 8.0) was heated at 100°C for 3 min, then slowly cooled to room temperature. The annealed and unannealed primers were separated using a Sepharose CL-4B column (0.7 x 20 cm) and 10 mM Tris-HCl (pH 7.5) - 1 mM EDTA as an eluent. The fractions containing primed template in the void volume were pooled and the primed template was ethanol precipitated. Polymerization was carried out in 5 ul of polymerization buffer containing template-primer (0.43 ug), Pol I Klenow fragment (1.3 units), and 10 uM each of 3 dNTPs or 3 dNTPs plus one modified nucleotide at 25°C for 40 min. Standard dideoxy sequencing reactions were also carried out according to the method of Sanger *et al.* (26). Samples were electrophoresed at 1500 V on a 8% denaturing polyacrylamide gel. The gels were autoradiographed using XAR-5 film at -70°C overnight.

RESULTS

Ability of DHdTTP and dTTP-GLY to serve as substrates for DNA polymerases

In order to examine the ability of DHdTTP and dTTP-GLY to replace dTTP as substrates for *Escherichia coli* DNA polymerase I (Pol I), Pol I Klenow fragment, T4 DNA polymerase, and AMV reverse transcriptase, a sequencing primer annealed to a M13mp11 template was elongated with the appropriate polymerase in the presence of three normal dNTPs (A,G,C) and a modified nucleotide. If DHdTTP or dTTP-GLY replaces the missing dTTP, elongation of the primer will occur, and the extent of the primer elongation as measured by [^{3}H]dCTP incorporation should reflect the rate of incorporation of the modified nucleotide. In accord with our previous results with Pol I Klenow fragment (8), elongation of the primer occurred in the presence of DHdTTP but not in the presence of dTTP-GLY, and the rate of primer elongation with DHdTTP was about 13-fold less than with dTTP (Fig. 2A). Essentially the same results were obtained with Pol I (Fig. 2A). Using a similar assay, we also found that Pol I used DHdTTP but not dTTP-GLY as a substrate in the nick translation of

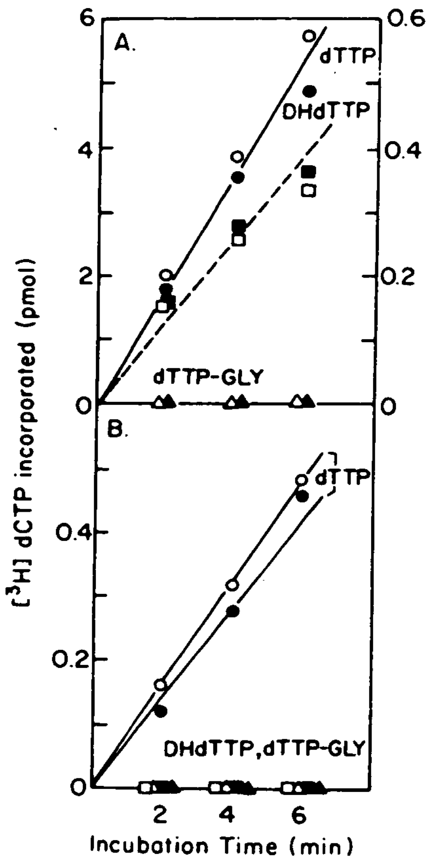

Figure 2. A. Primer elongation catalyzed by Pol I (open symbols) and Pol I Klenow fragment (closed symbols) in the presence of (○,●) dTTP, (□,■) DHdTTP and (△,▲) dTTP-GLY. The data for incorporation of DHdTTP and dTTP-GLY are shown by the units on the right axis. B. Primer elongation catalyzed by T4 DNA polymerase (open symbols) and AMV reverse transcriptase (closed symbols) in the presence of (○,●) dTTP, (□,■) DHdTTP and (△,▲) dTTP-GLY. The primed template (0.77 ug) in polymerization buffer (15 ul, MATERIALS AND METHODS) was replicated with 1.5 units of Pol I or Pol I Klenow fragment, 3 units of T4 DNA polymerase, or 6.2 units of AMV reverse transcriptase in the presence of 10 uM each of 3 normal nucleotides (dATP,dGTP,[^{3}H]dCTP) and 10 uM dTTP or a modified nucleotide at 25°C. The extent of primer elongation as measured by [^{3}H]dCTP incorporation was corrected by subtraction of the background obtained in a polymerization reaction with 3 normal nucleotides alone (dATP,dGTP,dCTP).

duplex PM2 DNA (data not shown). The PM2 DNA nick translated with DHdTTP gave positive signal with the antibody specifically elicited to dihydrothymine (Hubbard *et al*. personal communication). T4 DNA polymerase, which contains an active 3′-5′ exonuclease (proofreading) activity (27), used neither DHdTTP nor dTTP-GLY as substrates. Elongation of the primer was observed only with dTTP (Fig. 2B). Since the original purpose of this experiment was to engineer modified nucleotides into DNA, and since neither Pol I nor T4 DNA polymerase used dTTP-GLY as a substrate, we tested the ability of AMV reverse transcriptase to catalyze incorporation of the modified nucleotides into DNA. AMV reverse transcriptase has been shown to have reduced fidelity compared to the prokaryotic polymerases presumably due to its lack of 3′-5′ exonuclease

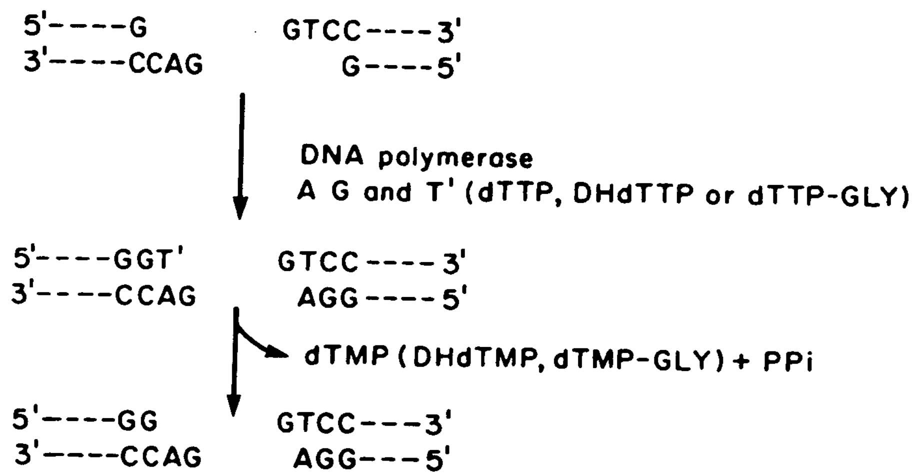

Figure 3. Schematic representation of DNA polymerase-catalyzed idling-turnover of dTTP or its analogues (T′) on an *AvaII*-digested PM2 DNA end.

(proofreading) activity (28). In contrast to our expectations, AMV reverse transcriptase did not use dTTP-GLY as a substrate. Even more surprisingly, DHdTTP, which was a substrate for Pol I, was not a substrate for AMV reverse transcriptase (Fig. 2B). Since with T4 DNA polymerase or AMV reverse transcriptase, the net dNTP incorporation in control experiments using dTTP was considerably lower than with Pol I (about 10-fold), we would not have detected DHdTTP or dTTP-GLY incorporation if they had been utilized with an efficiency less than 1/100 of dTTP. However, it should be also pointed out that the conclusions with T4 DNA polymerase were further supported by turnover experiments described below.

Turnover of DHdTTP and dTTP-GLY

In the polymerization reaction, dTTP, DHdTTP and dTTP-GLY were differentially discriminated by different DNA polymerases. Two distinct mechanisms that operate at the insertion or proofreading step could account for this observation. First, the substrates could be kinetically discriminated based on their K_m and V_{max} values at the insertion step. Secondly, the 3′-5′ exonuclease activities of Pol I and T4 DNA polymerase could have different specificities for inserted DHdTMP and dTMP-GLY. Since AMV reverse transcriptase has no associated 3′-5′ exonuclease function (28) and cannot remove inserted nucleotides, it must not be able to bind to the modified nucleotide triphosphates. In order to address the question as to

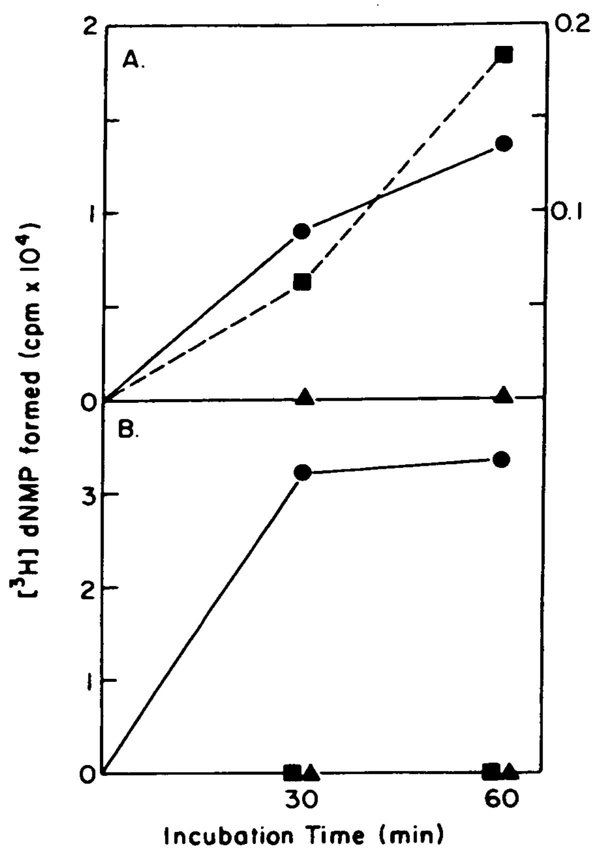

Figure 4. Pol I (A) or T4 DNA polymerase (B) catalyzed idling-turnover of (●) dTTP, (■) DHdTTP and (▲) dTTP-GLY on *AvaII* digested PM2 DNA ends (see also Fig. 3). In panel A, the data for DHdTTP are depicted by the units on the right axis. The reaction was carried out in the polymerization buffer (12 ul, **MATERIAL AND METHODS**) containing digested PM2 DNA (0.5 ug), 1.5 units of Pol I Klenow fragment or 5 units of T4 DNA polymerase, 1 uM each of dATP, dGTP, and ^{3}H-labeled dTTP or its analogue. The released monophosphates were separated and quantitated by TLC as described in **MATERIAL AND METHODS**.

which mechanism was operating for Pol I and T4 DNA polymerase, we carried out a turnover assay of [^{3}H]dTTP, [^{3}H]DHdTTP and [^{3}H]dTTP-GLY using *AvaII* digested PM2 DNA ends as a template. dCTP, which would be incorporated following dTTP or its analogue (see Fig. 3), was omitted from the reaction mix to enhance the enzyme catalyzed idling turnover reaction opposite adenine. With Pol I Klenow fragment, enzyme catalyzed turnover of DHdTTP was observed, however, no

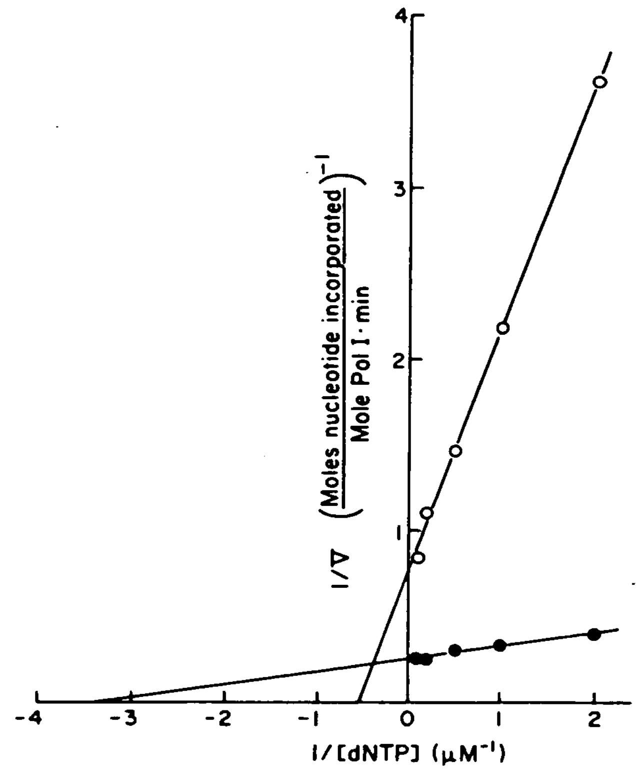

Figure 5. Double reciprocal plot of 1/V vs. 1/[dNTP] for the incorporation of (●) dTTP and (○) DHdTTP into poly(dA-dT) catalyzed by Pol I Klenow fragment. The initial rate (V) of DNA polymerization in the presence of [^{3}H]dATP (100 uM) and dTTP or DHdTTP ([dNTP] = 0.5-10 uM) was measured using Pol I Klenow fragment (2.5 units, 8220 units/mg) and poly(dA-dT) [10 ug, MW (1-5) x 10^5] as a template. The initial rate (V) was linear with incubation time (1,2,3 min) under these conditions.

turnover of dTTP-GLY was detected (Fig. 4A). The amount of released DHdTMP after 30 min reaction was 14-fold lower than that of dTMP obtained in a control experiment. The yield of [^{3}H]DHdTMP after 60 min incubation (1.8 x 10^3 cpm) was far above the upper limit (3.3 cpm) that could be accounted for by possible contamination of [^{3}H]dTTP in the [^{3}H]DHdTTP preparation. With T4 DNA polymerase, no turnover of DHdTTP or dTTP-GLY was observed, although dTTP was efficiently converted to dTMP by the idling reaction (Fig. 4B). The fact that the ability of the modified nucleotides to undergo enzyme-catalyzed

Table 1. Kinetic data for the incorporation of dTTP and DHdTTP into poly (dA-dT).

Substrate	K_m (uM)	V_{max}^{*1}	V_{max}^{*}/K_m^{1}
dTTP	0.30	1	19
DHdTTP	1.90	0.33	1

[1] Relative values.

turnover paralleled their ability to serve as substrates for polymerization (Fig. 2) strongly suggests that the efficiency of modified dTTPs to serve as substrates is determined at the insertion not the proofreading step.

Kinetic parameters for incorporation of DHdTTP with Pol I

Further insight into the substrate selection by Pol I Klenow fragment was obtained by measuring the initial rate of incorporation of $[^3H]$dATP into poly(dA-dT) in the presence of dTTP or DHdTTP. The rate of polymerization was linear with time and proportional to the enzyme concentration under the conditions used (data not shown). The kinetic parameters [K_m and relative V_{max} (V_{max}^*)] were calculated from double reciprocal plots of the data (1/V vs. 1/[dNTP]) using linear regression analysis (Fig. 5 and Table 1). The K_m for DHdTTP was 6.3 times higher than that for dTTP, suggesting that the binding affinity of DHdTTP to the Pol I-primer terminus complex is considerably weaker than that of dTTP. In addition, it is noteworthy that the lower V_{max}^* of DHdTTP relative to dTTP further reduces its efficiency as a substrate for Pol I. Based on the relative V_{max}^*/K_m values, DHdTTP should be about 19-fold less efficiently used as a substrate by Pol I than dTTP. This agrees fairly well with the results obtained in the M13 system described above.

Base pairing properties of the modified nucleotides

The ability of the modified nucleotides to replace any of the 4 normal dNTPs was tested using the technique of Revich *et al.* (25). This method is highly sensitive and the extent of primer elongation due to incorporation of a modified nucleotide can be resolved at the sequence level. Pol I Klenow fragment was used throughout this assay. In the minus A, G, and C reactions, where the mentioned nucleotide was missing from the reaction mix, no difference in the primer elongation was observed with or without DHdTTP (data not shown). However, the primer was significantly elongated over background

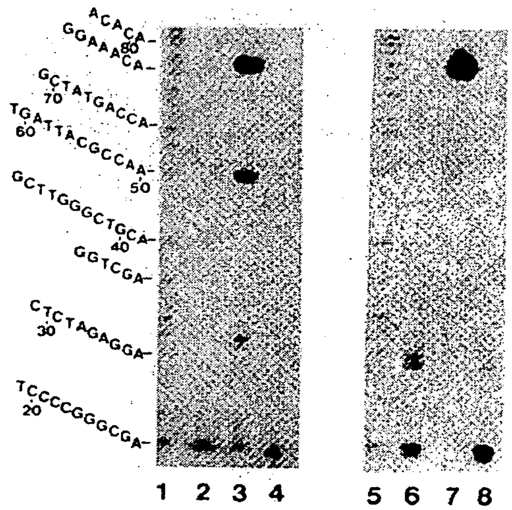

Figure 6. Polyacrylamide gel analysis of DHdTTP and dTTP-GLY incorporation during primer elongation catalyzed by Pol I Klenow fragment. M13mp11 template-primer (5'-end labeled) was replicated by Pol I Klenow fragment (1.3 units) in the presence of 10 uM each of 3 normal nucleotides (dATP,dGTP,dCTP) without (lanes 2 and 6) or with 10 uM DHdTTP (lanes 3 and 7) or 10 uM dTTP-GLY (lanes 4 and 8). The reactions were carried out with Mg^{2+} (lanes 2-4) or with Mn^{2+} (lanes 6-8). Lanes 1 and 5 show standard dideoxy T ladders. Also shown is the sequence of the template and the nucleotide positions from the primer terminus. Multiple adenine sites are present at the positions 50-51 and 74-76 in the template.

in the minus T reaction containing DHdTTP (Fig. 6, lane 3). These results clearly indicate that DHdTTP replaced only dTTP of 4 normal dNTPs. Similar experiments with dTTP-GLY confirmed that dTTP-GLY did not replace any of the 4 normal dNTPs. An example of a minus T reaction plus dTTP-GLY is shown in lane 4 of Fig. 6. We also examined the effect of substitution of Mn^{2+} (0.5 mM) for Mg^{2+} as the divalent cation. Mn^{2+} is known to reduce the fidelity of DNA synthesis (29,30,31). The patterns of the background primer elongation in the minus A, G, and C reactions were changed by substitution of Mn^{2+} for Mg^{2+}, however, the addition of DHdTTP or dTTP-GLY to the reaction mix did not further affect the patterns (data not shown). In the presence of Mn^{2+}, the elongation of the primer over background occurred only in the minus T reaction with DHdTTP (Fig. 6, lane 7).

Sequence specificity of DHdTTP incorporation

The analysis of the reaction products by high resolution sequencing gels revealed not only base pairing specificity (mentioned above) but also sequence

dependence of DHdTTP incorporation. As shown in lane 3 of Fig. 6, DNA synthesis catalyzed by Pol I Klenow fragment in the presence of Mg^{2+} passed most of the adenine sites in the template (or sites where dTTP should be incorporated). The positions of adenine in the template are indicated by the dideoxy T ladders in lane 1. However, strong termination bands of DNA synthesis appeared opposite clusters of adenine in the template (positions 50-51, 74-76), suggesting that multiple incorporation of DHdTTP led to the arrest of DNA synthesis. These termination bands were not observed in control polymerization reactions with 4 normal dNTPs and Mg^{2+} or Mn^{2+}, and only highly polymerized products were observed close to the well (data not shown). The substitution of Mn^{2+} for Mg^{2+} relaxed the fidelity of DNA synthesis so that the termination bands (positions 11-25, 50-51) observed with Mg^{2+} disappeared almost completely (Fig. 6, lane 7). However, under these conditions, strong termination bands, due to the arrest of DNA synthesis, were still observed opposite a triplet adenine site (positions 74-76).

DISCUSSION

Saturation of the C5-C6 bond in dihydrothymine and thymine glycol not only increases the length of this bond (1.35 Å - 1.52 Å) but also increases the length of adjacent C4-C5 and C6-N1 bonds (12). In addition, the thymine ring of these compounds is no longer planar and assumes a half chair conformation with C5 and C6 significantly out of the plane of the other four atoms. These lesions in damaged DNA are also removed by common repair enzymes such as *Escherichia coli* endonuclease III (32,33,34) and *Micrococcus luteus* γ-endonuclease (35,36). In this study we have shown that DNA polymerases can differentially recognize the alteration of the thymine ring in DHdTTP and dTTP-GLY as measured by enzyme catalyzed incorporation into DNA. The results of the turnover experiments using dTTP, DHdTTP and dTTP-GLY, and the kinetic parameters for the incorporation of DHdTTP with Pol I suggest that the difference in the ability of these nucleotides to serve as substrates for DNA polymerases is not due to specific excision of the inserted nucleotide (3′-5′ exonuclease activity) but rather the binding affinity (or K_m) and possibly V_{max} of the nucleotide. It is tempting to speculate that with Pol I, two structural factors could affect the kinetic parameters of the triphosphates. 1) the loss of aromatic character and concomitant distortion of the thymine ring which are common to both DHdTTP and dTTP-GLY, 2) the substituent groups at the C5 and C6 positions (H or OH). The loss of aromatic character and

concomitant distortion of the thymine ring in DHdTTP resulted in increased K_m and reduced V_{max} relative to those of dTTP (Table 1). Substitution of H atoms at C5 and C6 by the polar OH group in dTTP-GLY must have further affected the parameter(s) so that no incorporation or turnover of dTTP-GLY was observed.

Although the molecular mechanism of template-directed base selection and high fidelity of enzymatic DNA synthesis is not fully understood, the recent determination of the primary amino acid sequence of Pol I (37), the identification of the amino acid sequence of the deoxyribonucleoside triphosphate (dNTP) binding site (38), together with the information from NMR studies about the interaction of dNTP with Pol I (39,40), have greatly facilitated such mechanistic studies. Interestingly, dNTP substrates appear to bind to the dNTP binding domain of Pol I through two different types of interaction. There is a hydrophobic interaction between Ile-Tyr (residues 765 and 766) and the base moiety of the dNTP, and an ionic interaction between Lys (residue 758) and the metal chelated phosphate group of the dNTP. The numbers of the amino acids correspond to the primary sequence of the residues of Pol I reported by Joyce *et al.* (37). In this study we have found that the efficiency of dTTP derivatives as substrates for Pol I (dTTP > DHdTTP > dTTP-GLY) correlates well with the expected strength of the hydrophobic interactions between Ile-Tyr residues present in the dNTP binding domain of Pol I and the base moieties of the dTTP derivatives (hydrophobicity of the bases decreases in the following order: thymine > dihydrothymine > thymine glycol). Presumably, such an altered hydrophobic interaction led to the change in kinetic parameter(s) of the modified dTTPs, thereby reducing the efficiency of the modified dTTPs to serve as substrates for Pol I. It can be inferred that with T4 DNA polymerase and AMV reverse transcriptase, the loss of aromatic character and distortion of the thymine ring resulted in a tremendous change in the kinetic parameter(s) since no incorporation or turnover (T4 DNA polymerase) of DHdTTP or dTTP-GLY was observed (Figs. 2B and 4B).

With respect to the present *in vitro* results, it is noteworthy that we have found that exogenously added dihydrothymidine was incorporated into the DNA of PM2 phage (41), fl phage and the *Escherichia coli* chromosome (Ide *et al.* unpublished data), suggesting that dihydrothymidine can be phosphorylated to DHdTTP by kinases and incorporated into DNA by DNA polymerases *in vivo*.

In the presence of DHdTTP, the DNA synthesis catalyzed by Pol I passed most of adenine sites in the template (or where DHdTTP was incorporated), except where multiple adenine sites were present (positions 50-51 and 74-76,

Fig. 6, lane 3). Very weak termination bands were also observed at two single adenine residues for unknown reasons (positions 11-25). These data on the sequence specificity of DHdTTP incorporation indicate that, irrespective of the distortion in the thymine ring, the presence of dihydrothymine in the primer terminus site does not produce enough disorder to inhibit subsequent polymerization. However, multiple incorporation of DHdTTP residues in the newly synthesized strand, such as at 50-51 (doublet) or 74-76 (triplet), might produce enough strain to inhibit polymerization at these sites. This is probably due to hindered vertical (or stacking) interactions between the distorted thymine rings. In the presence of Mn^{2+}, which is known to reduce fidelity of DNA synthesis (29,30,31), the doublet but not the triplet dihydrothymine site was bypassed. This result can be explained by the presence of more disorder at the triplet site than at the doublet site. It should be noted that the observed arrest of DNA synthesis in the presence of Mg^{2+} or Mn^{2+} was not absolute but partial. If synthesis had been absolutely arrested at one site, only one termination band would have been observed on the gel. Since we have found that the interactions of dihydrothymine with adjacent normal bases in the same strand (stacking), and with the opposite base in the other strand (hydrogen bonding) were not significantly altered, it seems reasonable to predict that, in contrast to thymine glycol which has been shown to constitute a replicative block *in vitro* (14-17), dihydrothymine sites in the template strand should not constitute strong replicative blocks unless they are multiple dihydrothymine sites. In addition, neither lesion [dihydrothymine (this work) and thymine glycol (14-17)] should be strong premutagenic lesions since they appear to retain the ability to pair with the proper base, adenine. This prediction has been confirmed for thymine glycol lesions using OsO_4-oxidized M13 lacZ(α) hybrid phage (18).

Finally, the following results are noteworthy in connection with the fidelity of enzyme-catalyzed DNA synthesis. First, Goodman and co-workers have pointed out that the relative affinity (K_m) of dNTP for DNA polymerase forming a complex with a primer terminus is primarily responsible for the fidelity of the DNA synthesis (42,43,44). In other words, fidelity is mainly determined by the relative residence times of competing correct vs. incorrect dNTPs at the dNTP binding domain. Secondly, it has been shown that certain alkylated nucleotides (9,10,11) and nucleotide analogues (25,45-48) serve as substrates for DNA polymerases. However, in these cases, most of the modifications were introduced into the exocyclic functional group of the pyrimidine or purine bases which does not alter the planarity and aromatic

character of the bases. The results of the present study using DHdTTP and dTTP-GLY should provide further insight into the fidelity of DNA synthesis especially as it pertains to selectivity of the substrate molecule by different polymerases.

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